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Evaluation of angiogenesis in diabetic lower limb wound healing using a natural medicine: A quantitative approach

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ABSTRACT

Increasing incidents of diabetes (mellitus) induced non-healing lower extremity wounds and disease associated amputations have raised significant concerns related to quality of life of afflicted patients. High glucose level in diabetic wounds inhibits the transactivation of angiogenesis related molecules resulting delayed healing progression. Present study investigates the impact of a natural medicine like honey in angiogenesis of non-healing diabetic lower limb wounds. Quantitative assessment of different vessel parameters was performed on *in vitro* CAM model for validation of angiogenic potential of honey. Further the upregulation of angiogenesis related prime molecular markers like HIF-1α, VEGFA, VEGFR2 is under the therapeutic intervention of honey indicated improved angiogenesis which in turn promote the healing rate. These results may facilitate in determining the healing impact of this natural product in treatment of diabetic wounds and it may also help in developing alternative cost effective therapeutic modality.

1. Introduction

Successful management of diabetic chronic foot ulceration is an unmet clinical challenge and often leads to disease-associated amputations. In diabetic patients, impaired healing process has been attributed to micro and macro vascular alterations causes peripheral neuropathy and tissue hypoxia while abnormalities in inflammatory pathways lead to development of infectious non-healing foot wounds [1]. In such situations, besides anti-bacterial activity, stimulation of angiogenesis becomes necessary pre-requisite to promote adequate healing. Though hypoxic chronic wound ambience induces hypoxic-inducible factors-α (HIFα) that stimulates new blood vessels formation, high glucose level in diabetic wound prevents transactivation of HIF-1α and impairs angiogenesis by blocking the transcription of cascade of angiogenic activators like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stromal-derived factor-1(SDF-1), *etc.* [2,3]. Dysfunction of HIF-1α is majorly caused by reactive oxygen species which modify its co-activator p300 [4].

In wound beds angiogenesis is mainly controlled by the balance between angiogenic (like VEGF) and anti-angiogenic factors (like endostatin) [5]. VEGF signaling is a critical rate limiting step in sustained neo-vascularization. Its isoforms especially VEGF-A stimulates the angiogenesis process in paracrine manner after cutaneous injury and have been detected on the blood vessels of newly formed granulation tissues [6]. VEGF signaling is interceded by activation of transmembrane tyrosine kinase receptors – VEGFR-1 and VEGFR-2. During angiogenesis VEGF binds to VEGFR-2 leading to formation of new vasculature [7].

To manage chronic diabetes complications, different therapeutic strategies have been employed [8–10]. However, no single therapy have been shown to adequately support the multi-dimensional requirements of diabetic wound beds. In this direction, there is relentless search for appropriate therapeutic modalities that promote rapid healing as well as substantially provide a cost effective holistic support. Different alternative approaches for diabetes therapy includes various herbal preparations, dietary components and other natural products [11]. Amongst them, honey has been used as natural therapeutic agents as wound dressing for over 100 years. Its healing role as a topical agent for both infected and non-infected wounds [12] has been reported. In last few years, increased evidence-based reports on

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beneficial effects of honey encourage its therapeutic applications in diverse disease conditions including diabetes mellitus [13]. Amongst other therapeutic activities of honey, broad spectrum antimicrobial effects of honey are variably ascribed to acidic pH (3.2–4.5), H_2O_2 content and osmotic properties in addition to role of other components in low doses [14,15]. Different *in vitro* as well as *in vivo* studies have revealed interesting results that administration of honey along with other anti-diabetic drugs (like glibenclamide or metformin) can more efficiently reduce the serum glucose level in diabetic rats [16]. Moreover, honey is known to substantially decrease the serum fructosamine concentration which is not observed with standard anti-diabetic drugs. Combination of honey and anti-diabetic drugs are shown to improve the antioxidant defenses and reduce oxidative damage [17] which has impact in angiogenesis. Hydrogen peroxide present in honey plays stimulatory role in angiogenic which further facilitates granulation tissue formation. Cumulative effect of low pH and angiogenesis help to release oxygen in wound bed that stimulates tissue regeneration process [14,18]. Clinical evidences confirm rapid healing impact of honey with substantial angiogenesis role in diabetic wound repair process. However lack of scientific validations limits its application in contemporary medicine. Though some recent *in vitro* and *in vivo* studies have been scientifically interpreted the healing mechanism of honey but as the molecular basis mainly examined in animal models, they have a limited translational potential. Moreover, as origin of honey is a vital issue for quality control and to ensure consistent healing outcome, hence quantitative validation is necessary for developing a standard assessment process. This study intends to bridge the gaps between experimental outcome and clinical observations through quantitatively analyzing the different aspects of angiogenesis and expressions of related molecular markers under honey in lower limb diabetic wounds.

In this current study we sought to evaluate the angiogenic potential of physico-chemically characterized honey on treatment of non-healing diabetic foot wounds. In order to examine the vascular adaptation of this therapeutic agent, in *vitro* CAM assay has been performed and quantitative evaluation of vessel structure may predict the stimulatory role of honey in neo-vascularization. Further the modulation in the expressions of angiogenesis related prime molecules (like HIF1α, VEGF-A, VEGFR-2) during healing of diabetic wounds under therapeutic intervention of honey may provide scientific validation of its well known healing potential. However, further studies are required to elucidate in depth molecular mechanism that may answer the angiogenesis paradox of diabetic wounds and to establish therapeutic impact of honey in diabetic wound healing.

2. Materials and methods

2.1. Collection of honey

Honey (ripe and dark amber color honey) collected from bee keepers of greater Kolkata, India [19].

2.2. Physico-chemical characterization

2.2.1. pH, electrical conductivity and water content assessment

Electrical conductance and pH of honey was measured at 37 °C temperature with pH-conductivity meter (420A, Orion, UK). For pH measurement ROSS ultra glass combination glass electrode was used (Orion 8102BNUWP). Electrical conductivity was determined through Orion DuraProbe 4-Electrode Conductivity Cells (013605MD) with 0.55 cm^{-1} cell constant.

To measure the free water content, frozen honey was vacuum dried by lyophilizer (Laboratory freeze dryer, IIC Indus. Corp.). Water loss of the sample was measured at different time intervals (*i.e.*, 30, 60, 90, 120 min) up to the constant weight reached.

2.2.2. Estimation of total phenolic content

Total phenolic content in honey was determined by the method using Follin–Ciocalteu's phenol reagent (Fluka analytical, Germany). Aqueous solution of raw honey (*i.e.*, 5 g of honey dissolved in 50 ml of distilled water) was initially filtered (millipore filter −22 μm) and 500 μl of diluted honey was treated with 2.5 ml of Follin–Ciocalteu's phenol reagent (0.2 N) for 5 min. 2 ml sodium carbonate solution (75 g/l) was added to the above mixture and incubated for 2 h at room temperature in dark [20]. Absorbance at 760 nm was measured through UV–vis spectrophotometer (V-1601, UV-Visible Spectrophotometer, Shimadzu, Japan) against the blank methanol without honey. The concentration of phenolic compound was determined from the standard curve of gallic acid.

2.2.3. Estimation of catalase enzyme activity (H_2O_2 activity) and DPPH radical scavenging activity

For determination of catalase activity, 2 ml of 0.02 M hydrogen peroxide (H_2O_2) was added in 0.5 ml of honey dilutions (v/v in 2.5 ml PBS). 1 ml of this mixture was further mixed with 2 ml 5% potassium dichromate/acetic acid solution at time intervals of 0, 60 and 120 s. On addition of potassium dichromate, the enzyme begins to react with the honey forming a deep blue color and also bubbling of the liquid was seen due to the breakdown of hydrogen peroxide into hydrogen and oxygen. The solution was incubated in a boiling water bath for 10 min and OD was taken at an absorbance of 620 nm.

Radical scavenging activity was determined by 2,2-diphenyl-1-picryylhydrazyl (DPPH). 0.5 ml of different honey dilutions, 1 ml of methanol and 100 μl of DPPH was added to test tubes. Blank was prepared by adding 0.5 ml PBS, 1 ml of methanol and 100 μl of DPPH. The tubes were kept in the dark and absorbance was takes after 60 min at 517 nm in UV–visible spectrophotometer. Ascorbic acid was used as positive control. Changing of color indicates the reducing activity of DPPH to DPPHH. Yellow color indicated the scavenging efficiency of samples.

$$\text{Scavenging activity in } \% = A - B/A \times 100$$

A – is the absorbance of DPPH; B – is the absorbance of DPPH and honey combination.

2.3. Biological characterization

2.3.1. Anti-microbial activity

Bacterial strains like *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonae* and *Escherichia coli* were collected from B S Medical College, West Bengal, India. Disk diffusion test and minimum inhibitory concentration (MIC) assays were performed to determine the anti-bacterial activity of selected honey against these bacterial strains.

In disk diffusion technique, 100 μl of the standard bacterial nutrient broths of above mentioned isolates (cultures of the isolates at the density of 1.5×10^8 CFU/ml) were spread on Muller Hinton agar (HiMedia) plates. Honey impregnated filter paper disk (8 mm in diameter) was placed on each types of bacteria inoculated plates. Commercially available tetracycline disk (30 mcg) and sterilized distilled water were set as positive and negative control respectively. Plates were incubated at 37 °C for 24 and 48 h under aerobic condition. After incubation period, the inhibition zones around discs were measured in millimeters (mm). Tests were performed in triplicate with fresh subcultures.

In MIC assay, honey was serially diluted with de-ionized sterilized distilled water [concentration of 70%, 60%, 50%, 40%, 30%,

15%, 10% and 5% (v/v) honey in de-ionized sterilized distilled water]. Diluted solutions were initially screened against the bacterial strains by disk diffusion technique and the lowest concentration of honey that exhibited bacterial resistance was selected for MIC. This study was performed in triplicate.

2.3.2. Chorioallantoic membrane (CAM) analysis

A batch of fertilized chick eggs ($n = 60$) were washed with potassium permanganate and wiped with 70% alcohol and incubated at 37 °C with optimum humidity for 48 h. Afterwards, 2 ml of egg albumin was removed without disturbing the yolk and again incubated for 7 d at 37 °C. Eggs were divided into 4 equal groups *viz.* (a) positive control (treated with angiogenin factor – Angiogenin Human recombinant no. A6955, Sigma Aldrich), (b) negative control (treated with water), (c) raw honey. Discs (1 mm) containing the therapeutic agents were inserted inside the egg followed by 2 d of incubation.

Assessment of blood vessel formation under different study groups in CAM assay was performed using stereo zoom microscopy (Olympus MVX10, Japan) under 40× (NA 0.5). Images were grabbed digitally by DP-72 camera (Olympus, Japan) at 1360 × 1024 pixels.

2.4. Application of honey on non-healing diabetic foot wounds

2.4.1. Clinical study

Patients ($n = 6$) of either gender (age = 45–65 years) with non-healing lower extremity wounds (traumatic origin) having exudation of pus, foul smell, and necrotic tissues and non-responding to conventional topical antibiotics were included under informed written consent. Ethical clearance was obtained from institutional ethical committee according to Helsinki declaration.

Subsequently, honey based occlusive dressing (*i.e.*, honey-soaked gauge followed by a layer of dry cotton tied with crepe elastic bandage) was applied on LLW. Redressing was performed with an interval of 24 h for initial 7–8 d having foul odor, exudation and necrotic tissues in the wounds and with progression of healing interval increased to 48–72 h. Clinically, pain, malodor, edema, debridement, granulation tissue formation, and epithelialisation were recorded.

2.4.2. Inclusion and exclusion criteria

Six patients with type 2 diabetes of age 45–65 years; body weight – 80.2 ± 3.5 kg; BMI – 29.5 ± 1.9 kg/m^2; FPG, 170 ± 10 mg/dL; and diabetes duration, 2.5 ± 0.5 years were studied. Patients were suffered from non-healing lower extremity wounds and non-responding to conventional topical antibiotics (for soframycin) were included under informed written consent [21].

2.4.3. Collection of biopsies

Incisional biopsies from wound edge were collected from patients ($n = 6$) under local anesthesia (Xylocaine) before and after (*i.e.*, 15th and 22nd d) honey dressing. Normal skin samples collected from superfluous tissues of surgical interventions.

2.4.4. Tissue processing

Biopsies fixed with 10% phosphate buffered formalin and processed for 4 μm thick paraffin sections on poly-L-lysine (Cat. No. P 8920 Sigma–Aldrich, St. Louis, MO, USA) coated slides.

2.4.5. Immunohistochemistry

Tissue sections baked and deparaffinized then hydrated for antigen retrieval in 10 mM citrate buffer (pH 6.0) using EZ-Retriever System V.2 (Bio-Genex, San Ramon, California, USA) and immunostained with kit (*i.e.*, Super Sensitive Polymer-HRP IHC Detection System Cat. no: QD400-60K BioGenex). Sections incubated with primary antibodies (Anti-HIF1 alpha antibody [EP1215Y]-(ab51608); abcam; Anti-VEGF antibody [14–124]-(ab16883); Abcam; Anti-VEGF Receptor 2 antibody [SP123]-(ab115805); Abcam) in 1: 500 dilution. Primary antibody binding visualized using a horseradish peroxidise conjugated secondary antibody using the chromogen 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Appropriate controls were put up to validate the experiments.

2.4.6. Microscopic studies

Immunohistochemical assessments were performed using Zeiss Observer.Z1 Microscope (Carl Zeiss, Germany) under 20× (NA 0.8; pixel resolution 0.31) and 40× oil (NA 1.3; pixel resolution 0.16). Images were taken digitally by CCD camera (AxioCam MRC, Zeiss) at 1388 × 1040 pixels.

2.5. Image processing

2.5.1. Image analysis of CAM assay

Angiogenic features on CAM model were quantitatively evaluated for all three classes of interventions. Parameters like – (1) vascular area, length of vessel skeleton, (2) first order features like vessel branch point density and end point density and (3) vessel density (minor and major) were extracted through *via* appropriate image analytics techniques schematized in Fig. 1. Maximal-contrast green color channel was digitally extracted from the input CAM image and contrast was enhanced using contrast limited adaptive histogram equalization method [22]. Multi-scale second order Hessian based Frangi Vesselness filter was employed to selectively enhance the vessel like tubular structures including the minor vessels and suppresses avascular structures [23]. To preserve the inherent vessel structure, an edge-sensitive locally adaptive thresholding approach was used which also aided in

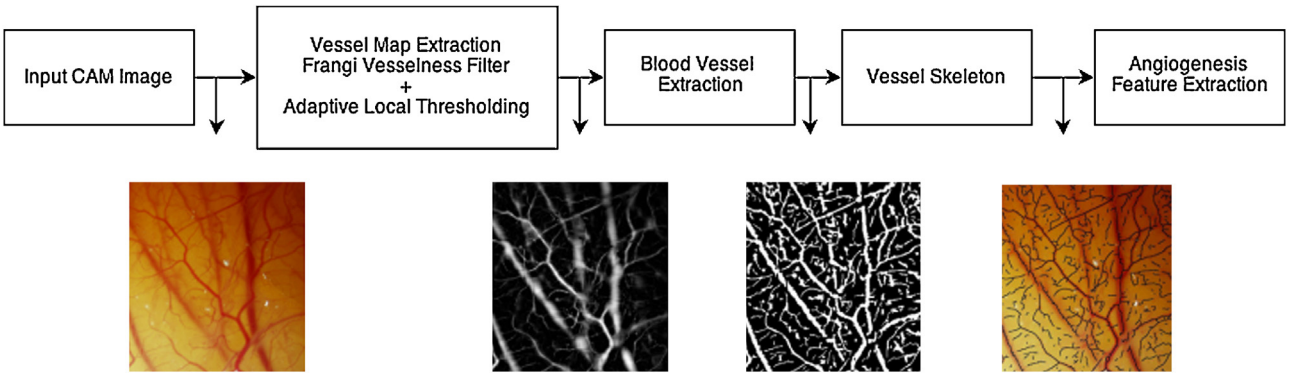

Fig. 1. Schema for image analytics and quantitative feature extraction from CAM images.

calibration and generation of the corresponding blood vessel binary image [24]. Finally, the blood vessel skeleton was extracted using Medial Axis Transform and spurious end points, however the non-vascular structures were iteratively pruned subsequently. Table 1 summarized the formula used in extraction of features quantifying global angiogenesis along with derived first order features from the vessel skeleton [25–28].

For analysis of IHC images (n = 14) for HIF1 alpha expression, the target features (image intensity, lengthwise distribution) of different groups (pre, post-intervention and normal) were segmented by *K*-mean clustering method. Each image was subsequently analyzed by their intensity values. And the length of spread of distribution from basement membrane to point of interest then measured for each group. Paired '*t*' test was performed to observe the significance level ($p < 0.05$) at different time points of healing.

3. Results and discussion

3.1. pH, electrical conductivity, water and phenolic content of honey and their impact in altering diabetic wound ambience

Indian honey under investigation exhibited acidic pH and conductivity ~18.39 μS/cm. This acidic pH of raw honey has potential impact on repair of non-healing/chronic wounds which have tendency to acquire alkaline ambience. Available literature reported that lowering the pH in chronic wound has strong influence on cellular, molecular cascades in wound bed consequently aiding progressive healing [29]. Further, this honey contains optimum amount of free water (~13%) and phenolic compound (~118 μg/ml) which stimulate its anti-bacterial activities. The viscous and hygroscopic nature of raw honey provides a protective barrier from various infections. Large amount of sugars along with minimum amount of free water in honey may also facilitate optimal moisture retention in wound bed.

3.2. Peroxide content and free radical scavenging activity of honey and their association with diabetic wound healing

H_2O_2 production in honey is due to the action of its inherent component glucose oxidase which is known to oxidize glucose to gluconic acid and produces peroxide as byproduct. Much of the literature indicated that H_2O_2 production is greatly increased with dilution of honey [30] and our current data (Fig. 2a) also indicated that catalase activity is highest at 20% (v/v) honey dilution. Generally dilution rate of honey depends on the amount of wound exudates. So it is important to know the concentration of H_2O_2 at different honey concentration. It has been found that with dilution, approximately 1–2 mmol/L H_2O_2 is produced and with this concentration the possibility of cyto-toxic damage in healing bed is very low [30].

During healing process of any wound types, cells generally enter from an quiescent state to highly proliferative and migratory state. Besides stimulatory role of different growth factors/cytokines, H_2O_2 in low dose is found to replicate the growth factor signaling especially related to cellular proliferation and migration. In addition to its well known antibacterial activity by stimulating body's inflammatory cells, H_2O_2 is also known to promote the angiogenesis by triggering important angiogenic factor like VEGF through oxidant induction meditated by activated neutrophils [31].

Now the question remains whether excessive ROS which is produced during healing process caused poor healing? Different studies on the expression of biomarkers related to oxidative damage indicated that ROS may cause damage if they are not properly alleviated by anti-oxidants. However, even with the incomplete knowledge regarding the impact of oxidative damage in healing process several studies reported the beneficial effect of anti-oxidants in diabetic wound healing [32]. Anti-oxidants that quench free radicals are generated in chronic inflammatory loop in diabetic wounds. Honey is a unique therapeutic agent which has anti-oxidant activity along with capability of producing low dose peroxide [33]. Anti-oxidant activity of honey was measured through its DPPH free radical scavenging activity (Fig. 2b). The % inhibition of free radical increases with honey dilutions from 60% to 5%. But in standard (ascorbic acid) the % inhibition of free radical decreases with dilutions. So in comparison to standard the % inhibition of free radical is inversely related to the honey dilutions.

It is believed that high level of ROS especially H_2O_2 (~166 mM) may caused prolonged inflammation and neutrofil associated proteolytic environment which leads to delayed healing process. However very low concentration of H_2O_2 may promote the healing process in diabetic wound through increasing cellular migration and angiogenesis [34]. Here the unique compositional balance of honey with low dose H_2O_2 production capability along with radical scavenging activity for excess ROS generated especially in non-healing wound may establish this natural healing agent as an important candidate for diabetic wound healing. However, in this regard more studies are required to unveil the exact molecular mechanism under honey intervention.

Table 1
Features evaluating angiogenic potential along with their mathematical formulation and physiological importance.

Name of angiogenic feature	Mathematical formulation	Physiological importance
% Vascular area	$\frac{\text{Total vessel area}}{\text{Total area of the image}}$	It measures the percent contribution of vascular and avascular tissues in the given field of view. It is a global measure quantifying the degree of vasoproliferative response of the angiogenic drug being administered.
Length of vessel skeleton	$N + (\sqrt{2}-1)N_d - 1$ where N = No. of pixels in vessel skeleton; N_d = No. of diagonal pixels in vessel skeleton	It quantifies the total length of the vascular tree in the field of view.
Vessel branch point density	$\frac{\text{No. of vessel branch points in field of view}}{\text{Total area of the field of view}} \times 10^4$	It is a first order derived feature from the vessel skeleton. It quantifies the degree of branching of the vascular network.
Vessel end point density	$\frac{\text{No of vessel end points in field of view}}{\text{Total area of the field of view}} \times 10^4$	It is a first order derived feature from the vessel skeleton. It quantifies the degree of transition of the vessels from vessels to the capillary plexus.
Major vessel density	$\frac{\text{Total no. of major vessels in field of view}}{\text{Total area of the image}} \times 10^4$	It quantifies the major micro-vessel density in the field of view (vessels >200 μm).
Minor vessel density	$\frac{\text{Total no. of minor vessels in field of view}}{\text{Total area of the image}} \times 10^4$	It quantifies the minor micro-vessel density in the field of view (vessels ≤200 μm).

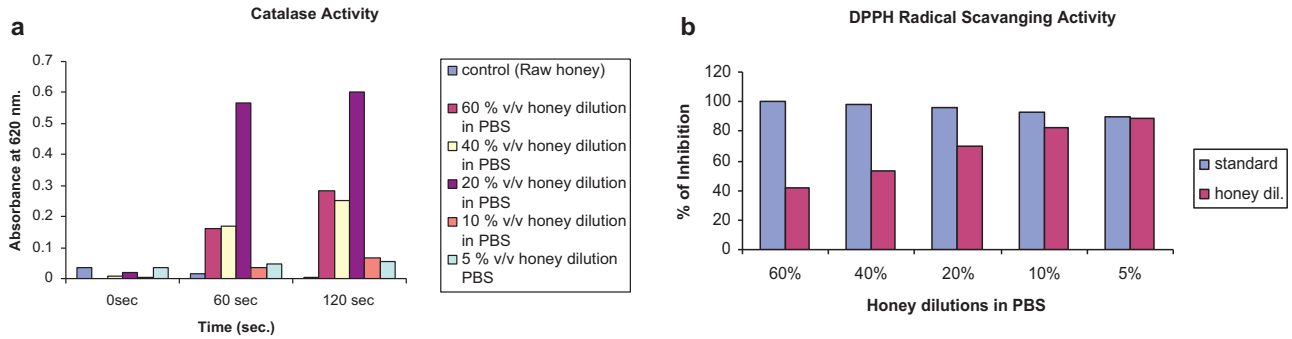

Fig. 2. (a) Catalase activity of different honey dilutions at different intervals, (b) DPPH radical scavenging activity of different honey dilutions.

3.3. Anti-microbial role of honey

Diabetes induced foot infections are serious problem often developed due to resistance to antibiotics. Moreover the bacterial proteases digests extra cellular matrix important for tissue regeneration. Outer coat of bacteria stimulates sever inflammatory response that often causes ulcerations by activation of the proteases. Most commonly occurring pathogenic strains in diabetic foot ulcer are *S. aureus*, *P. aeruginosa*, and Proteus [35]. Different studies revealed the broad spectrum antibacterial activity of honey against the most commonly encountered infections (like MRSA12, 21–24 and VRE12) that have been encountered during diabetes induced foot infections [36]. In present study, disk diffusion test revealed antibacterial properties of the Indian honey in terms of zone of inhibition against five different human pathogenic strains like *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumonae* and *S. epidermidis*. Honey although exhibited significant inhibition zone against the *S. aureus*, *E. coli*, and *P. aeruginosa* but no inhibition on *K. pneumonae*, *S. epidermidis*.

Further Minimum inhibitory concentration (MIC) study indicates that at 70%, 60%, 50% and 40% (v/v) concentrations honey shows inhibitory effects against the pathogens like *S. aureus*, *E. coli* and *P. aeruginosa* (Table 2). MIC assay revealed 5% concentration of the honey produced no antibacterial effect where at concentration of 10% a small inhibition zone against *S. aureus* and *E. coli* were found and for *P. aeruginosa*, 40% of honey produced a small inhibition zone.

3.4. Quantitative analysis of angiogenesis potential of honey on in vitro CAM model

CAM assay is an established reliable model to evaluate the pro- or anti-angiogenic potentials of various drugs/molecules. In the present study, vascularization was remarkably more (Fig. 3) under treatment of honey in comparison to negative control. Analysis of different parameters of angiogenesis in study groups depicted that under intervention, the mean vascular area under honey was increased by 75.54% with respect to negative control and by 15.05% with respect to positive control (Fig. 4). Total length of the vessel skeleton also increased two-fold under intervention when compared to negative control. These global features are indicative of the increased degree of angiogenesis under the intervention and overall enhanced vasoproliferative potential of honey.

In CAM study, the pattern of vasculature depends on the combined action of both deterministic and random processes. During development, initially an abundant capillary meshwork is formed with an increasing number of branches and bifurcations that connect with various capillary beds to their stem vessels. Vascular network formation proceeds along three main stages: (i) migration and early network formation; (ii) network remodeling and (iii) differentiation in tubular structures as well as the development of capillary networks. These capillary networks are further characterized by typical intercapillary distances ranging from 50 to 300 mm which is important for optimal metabolic exchanges. In general the complexity of CAM vasculature refers to the density of the blood vessels. However, under different molecular interventions, vascular complexity may change depending on the altered growth and distribution of vessels. Several studies have been performed to understand the logic of vascular network growth. Previously feature extraction from CAM images were tedious and have significant inter-observer variability due to the use of low magnification bright field images [28]. To overcome these shortcomings, digital image analysis packages for extraction of blood vessels and their quantification have been employed. In this study, robust and locally adaptive edge-sensitive vessel extraction algorithm has been proposed which handles variations in illumination [37].

Fig. 4 indicated that the factors related to vascular network development significantly increased under honey. Vessel branch point density increased by 81.20% and 22.64% with respect to negative control positive control respectively, while the corresponding vessel end point density exhibited 46.79% and 17.17% with respect to negative and positive control. Major vessels density also increased 2.6 times in comparison to the negative control. Corresponding minor vessels observed 77% in increase in their density with respect to the negative control and 20.3% increase with respect to the positive control. All together the results indicate remarkable angiogenic potential of honey that may stimulate the rapid healing progression of non-healing diabetic wounds.

Table 2
Minimum inhibitory concentration of honey.

Honey sample	Zone of Inhibition (in mm)		
	S. aureus	*E. coli*	*P. aeruginosa*
70%	22.9 ± 0.1	23.1 ± 0.3	16.6 ± 0.5
60%	20.9 ± 0.8	22.4 ± 0.4	13.3 ± 0.5
50%	20.2 ± 0.4	22.0 ± 0.1	8 ± 0.1
40%	19.0 ± 0.1	17.3 ± 0.3	5.1 ± 0.1
30%	8.9 ± 0.1	14.9 ± 0.1	ND
15%	7.0 ± 0.1	14.2 ± 0.4	ND
10%	5 ± 0.1	8.1 ± 0.2	ND
5%	ND	ND	ND
H_2O	ND	ND	ND

3.5. Honey modulates the expressions of angiogenesis related markers during lower extremity wound healing

HIF-1α plays a crucial role in stimulating multiple angiogenic growth factors [14] in chronic wound ambience. Different studies have shown hyperglycemia in diabetic wound impairs the function

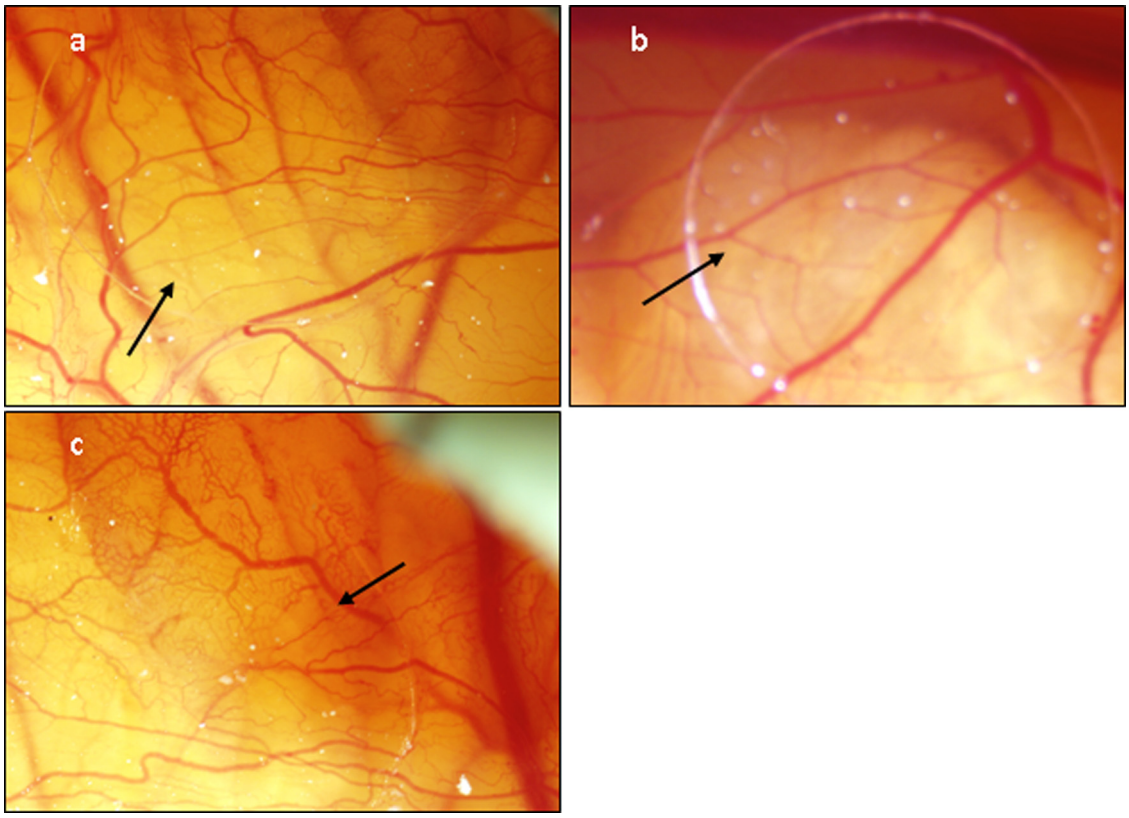

Fig. 3. Stereo-zoom microscopic images (40×) of angiogenesis in CAM development under (a) positive control (b) negative control (c) honey. Arrow indicated healing agent impregnated disk.

and stability of this molecule [2]. Accordingly in present study, expression of HIF-1α was found to be significantly ($p < 0.001$) low in pre intervention biopsies (5c) in comparison to 15 days post intervention (5b). Expression of this molecule was substantially increased both in terms of intensity as well as length of spread of distribution from basement membrane in post intervention samples (data not shown). Whereas, in normal tissue expression level was comparably low than post intervention biopsies

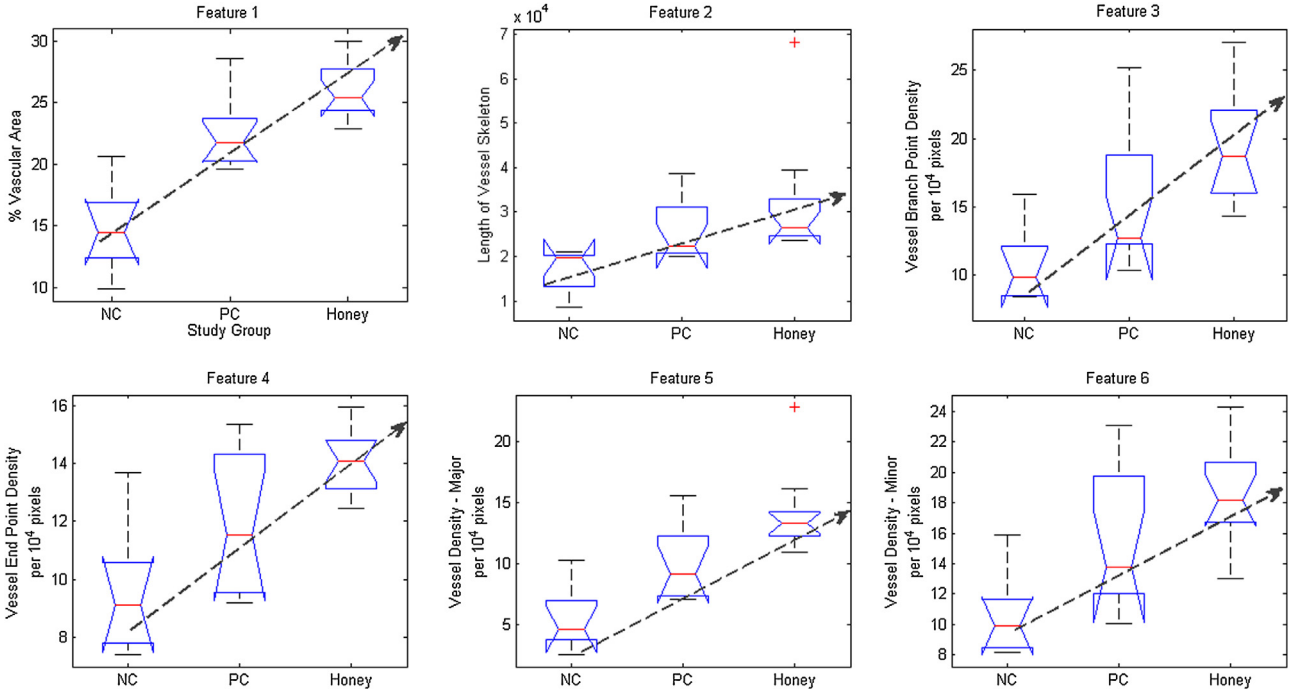

Fig. 4. Notch box plots of quantitative features for assessment of CAM assay depicting their trends under different interventional agents. These are (a) % vascular area, (b) length of vessel skeleton, (c) vessel branch point density, (d) vessel end point density, (e) major vessel density and (f) minor vessel density. The increasing trend of these features indicates increasing pro-angiogenic potential and increased complexity and extent of the underlying vascular structures.

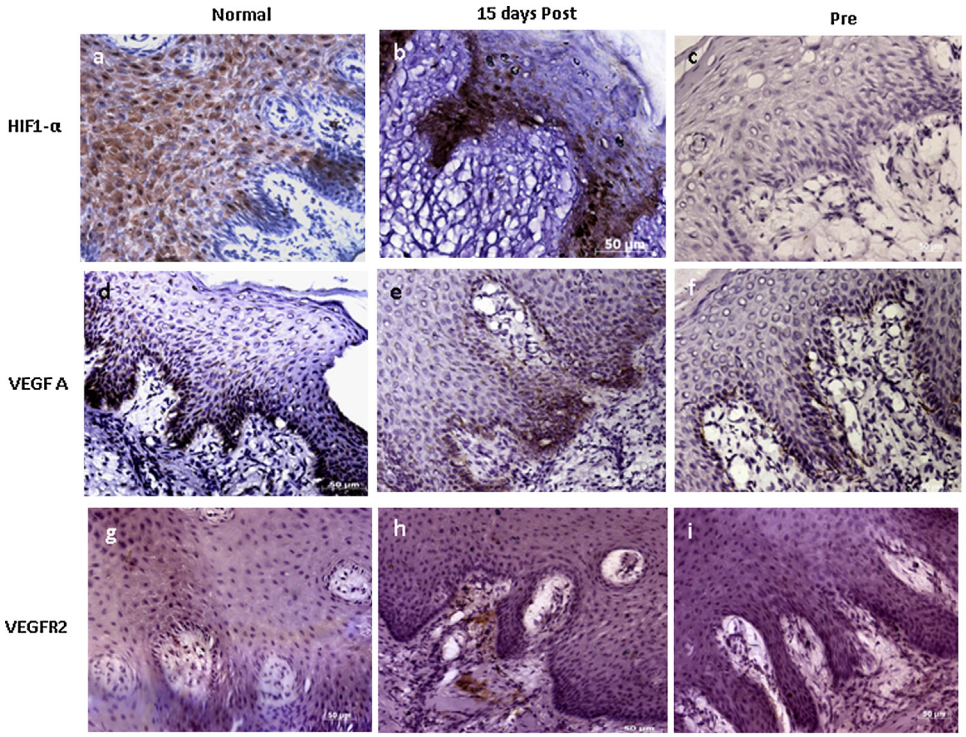

Fig. 5. Immunohistochemical photomicrographs (20×) of skin biopsies (normal and leg wound periphery): (a)–(c) depicted expression of HIF1α in normal (a), before (b), 15th days after (c) topical intervention of honey: (d)–(f) demonstrated VEGFA expression in normal (d) skin and in peripheral biopsies of before (e) and after said days of interventions (f); (g)–(i) depicted expression of VEGFR2 in normal skin (g), pre-intervention (h) and post-intervention (i). Expressions of HIF1α, VEGFA and VEGFR2 substantially low in pre intervention sample (a, d, g) in comparison to post intervention and normal biopsies.

indicated the absence of chronic ambience (Fig. 5a). Several studies have reported the down regulation of HIF-1α in diabetic wounds resulted due to inhibition of transactivation of this molecule under hyperglycemic ambience that specifically impair binding of ' HIF-1alpha to the coactivator p300 [39]. Although detail molecular mechanisms underlying impairment of HIF-1α in diabetes is poorly understood but some recent studies predict that high glucose ambience augments oxidative stress and stimulates excess ROS generation that in turn affects HIF-1α regulation [39]. Application of different hydroxylase inhibitors, (DMOG, DFX, *etc.*) with anti-oxidant activity has been studied to stabilize and activate HIF-1 α in diabetic wounds [2]. Here, anti-oxidant components of honey and their radical scavenging activity possibly help in structural and functional stability of HIF-1α in diabetic wound ambience.

Inhibition of transactivation of HIF-1α also inhibits another prime angiogenesis factor – VEGFA which is an essential mediator of neovascularization during healing process [38]. In pre-intervention tissues (Fig. 5b), expression of VEGFA is substantially low and mostly confined in the basal region whereas in 15 days post intervention (Fig. 5c), expression level is remarkably increased. Expression of VEGFR2 (Fig. 5g–i) is also improved in post-intervention biopsies (Fig. 5i) which is almost not detectable in pre-intervention sample (Fig. 5h). These observations show a positive corroboration with HIF-1α expressions and it may indicate that with application of honey the hyperglycemic environment in diabetic wounds is getting modulated. Recent report by Erejuwa O. hypothesized an interesting fact that besides anti-oxidant role, other components of this sugar-rich product especially fructose and oligosaccharides might have some hypoglycemic effect [40]. In addition to its inhibitory role on oxidative stress and hyperglycemia, honey ameliorates other metabolic disorders like hepatic trans-aminases, triglycerides, HDL cholesterol, *etc.* [41].

Though application of sugar-rich material like honey (almost 70% of its composition) in diabetic wounds is a bit starling however, beside high content of sugars, anti-microbial and radical scavenging anti-oxidant activities of honey have a promising role in quenching of excessive ROS produced in non healing diabetic wounds which stall the healing progression. Interestingly, different oligosaccharides and fructose components of this natural healing agent have been reported to contribute in reversing the hyperglycemic condition [40]. These budding evidences hypothesize that honey possible produce a conducive healing ambience and promote the pro-healing molecular cascades for successful repair of diabetic wounds. Present investigation tries to enlight the pro-angiogenic potential of honey and its impact on diabetic lower limb wound healing through modulating the angiogenesis related molecular events. However, more studies are needed to identify the component specific contribution of honey and related cellular/molecular signaling pathway that trigger the angiogenesis process.

4. Conclusion

Healing impact of honey in diabetic wounds has been reported. This study quantitatively depicted pro-angiogenic potential of honey on *in vitro* CAM model. This quantitative assessment may provide a standard for angiogenesis pattern of honey that can possibly differ with origin of honey samples and helps in quality control prior to clinical application. Further clinical study suggest that wound healing in subjects with type 2 diabetes was accelerated under honey dressing which modulate the expressions of prime angiogenic factors like HIF-1α, VEGFA, VEGFR2 which actually inhibited in hyperglycemic ambience in diabetic wounds.

Antioxidant content and radical scavenging activities of honey possibly play a crucial role in controlling over production ROS in diabetic wounds. Assessment of these molecular events evidenced the healing impact of honey in diabetic lower limb wounds by facilitating the angiogenesis events which otherwise stalled in hyperglycemic wound ambience.

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